Acta Derm Venereol (Stockh) 1998; 78: 169–172

Comparative Study of the *In vitro* Inflammatory Activity of Three Nickel Salts on Keratinocytes

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Nickel, the allergen of contact dermatitis, induces the *in vitro* production of inflammation markers such as intracellular adhesion molecule-1, interleukin-1 and tumour necrosis factor-α by keratinocytes. The purpose of our study was to compare the effect *in vitro* of different nickel salts on keratinocyte activation in order to determine whether this process depends on the nature of the salt used. Cultured keratinocytes were incubated with three nickel salts for 24 h in MCDB153 medium without hydrocortisone. Nickel gluconate, nickel sulphate and nickel chloride were each used at four concentrations: 5.10^{-5} M, 1.10^{-4} M, 5.10^{-4} M and 1.10^{-3} M. Keratinocyte activation was studied through the production of three inflammation markers: intracellular adhesion molecule-1, tumour necrosis factor-α and very late antigen-3 (an integrin with increased expression during contact dermatitis). Marker production was detected by immunocytochemistry and flow cytometry. Tumour necrosis factor-α production and intracellular adhesion molecule-1 and very late antigen-3 expression increased with addition of the three nickel salts, becoming maximal for nickel gluconate 1.10^{-4} M. In a subsequent experiment, zinc gluconate (an anti-inflammatory molecule) at 9 μg/ml reduced the very late antigen-3 expression induced by nickel gluconate 1.10^{-4} M. Therefore, this study enabled us to determine the concentration of a nickel salt (nickel gluconate) inducing optimal keratinocyte activation in our culture conditions and also indicated the potential interest of very late antigen-3 as an inflammation marker. *Key words: inflammation; skin.*

(Accepted November 17, 1997.)

Acta Derm Venereol (Stockh) 1998; 78: 169–172.

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Nickel is an allergen often responsible for contact dermatitis in the general population (1). Several *in vitro* studies of the inflammatory action of this metal on keratinocytes have shown that it induces the expression of certain adhesion molecules [e.g. intracellular adhesion molecule-1 (ICAM-1)] (2) and increases the production of inflammation cytokines such as interleukin-1 (IL-1) (2, 3) and tumour necrosis factor-alpha (TNF-α) (4). However, this effect may depend on the type of nickel salt used and its concentration, factors which have not been investigated in previous reports.

The purpose of this study was to compare the keratinocyte activation induced by three different nickel salts (nickel gluconate, sulphate and chlorine), used at four different concentrations, in order to determine the salt and the concentration inducing optimal keratinocyte activation. The modulations induced by nickel were studied not only by the classical markers of keratinocyte activation, i.e. TNF-α and ICAM-1, but also by the expression of very late activation antigen-3 (VLA-3), an integrin showing increased levels in certain forms of inflammatory dermatosis, particularly contact dermatitis (5). We also studied the modulation of VLA-3 expression induced by zinc gluconate, a substance with anti-inflammatory properties.

MATERIALS AND METHODS

Keratinocyte culture

Normal human keratinocytes extracted from healthy prepuces were cultured at 37°C in a humid atmosphere, in the presence of CO_2, in 25 cm^2 flasks (Nunc, Roskilde, Denmark) containing 7 ml of keratinocyte-SFM medium (Gibco BRL, Cergy-Pontoise, France) supplemented with 0.5 ng/ml EGF (Gibco), 25 μg/ml bovine pituitary gland extract (Gibco), penicillin 200 IU/ml-streptomycin 200 μg/ml (Boehringer-Mannheim, Meylan, France) and 0.25 μg/ml Fungizone (Bristol-Meyers Squibb, Paris, France). Forty-eight hours before addition of nickel salts, the cells were incubated in 6-well plates (Nunc) with 2 ml of low calcium (0.1 mM CACL2) defined-medium free of hydrocortisone MCDB153 (Biochrom KG, Berlin, Germany) and supplemented with 5 ng/ml EGF (Gibco), 50 μg/ml bovine pituitary gland extract (Gibco), 200 mM L-glutamine (Eurobio, Les Ulis, France), 5 μg/ml insulin (Sigma-Aldrich, Saint-Quentin Fallavier, France), 0.1 mM ethanolamine (Sigma), 0.1 mM phosphoethanolamine (Sigma), penicillin 200 IU/ml-streptomycin 200 μg/ml (Boehringer-Mannheim) and 0.25 mg/ml Fungizone (Bristol-Meyers Squibb). The cells were at 70% confluence in the wells when stimulated for 24 h with nickel gluconate or nickel sulphate ($NiSO_4$, $6H_2O$, Laboratoire Labcatal, Montrouge, France or nickel chloride ($NiCL_2$, $6H_2O$, Sigma). Each salt was used at the concentrations 5.10^{-5}M, 1.10^{-4} M, 5.10^{-4} M and 1.10^{-3} M. Culture medium without salt served as the control.

We also studied zinc-induced modulations of VLA-3 expression. In these experiments, keratinocytes were incubated with zinc gluconate (Laboratoire Labcatal) 0.9 and 9 μg/ml, which was added to the culture medium 24 h before incubation of the keratinocytes with the nickel salt determined during the preceding step.

For flow cytometry, experimental series were performed twice in duplicate. Student's *t*-test was used for the statistics.

Methods

Treated or untreated keratinocytes were detached from culture wells with trypsin 0.05%. After trypsin inactivation by fetal calf serum, the cells were centrifuged (400 × g for 10 min) and then washed in phosphate-buffered saline (PBS).

Immunocytochemistry

Keratinocytes (around 20,000) were fixed on poly-*l* lysined slides incubated for 30 min at room temperature first with a monoclonal antibody [anti-ICAM-1 mouse IgG 1 : 3, or anti-VLA3 mouse IgG 1 : 5 (Immunotech, Marseille, France), or anti-TNF-α mouse IgG 1 : 30 (Genzyme, Cambridge, MA)] and then, after washing in PBS, with a second antibody [an $F(ab)'_2$ fragment of goat mouse anti-IgG conjugated with fluorescein isothiocyanate (FITC) 1 : 15 (Caltag, San Francisco, CA), or with an $F(ab)'_2$ fragment of biotinylated goat anti-mouse anti-IgG 1 : 100 (Immunotech) and phycoerythrin-conjugated streptavidin 1 : 100

 ISSN 0001-5555

(Dako, Copenhagen, Denmark) for the detection of ICAM-1. Two negative controls were performed, one with an irrelevant mouse monoclonal antibody of the same isotype as the first antibody, and the other by omission of the first antibody.

The slides were read blindly (two independent investigators) in fluorescence microscopy (Leitz) in four different fields (magnification × 40). For each slide, a mean was determined semi-quantitatively for the four fields, using the following ratings: – (negative), + (low intensity), + + (moderate intensity), + + + and + + + + (high intensity).

Flow cytometry

Flow cytometry was performed in duplicate on aliquots of 200,000 cells. PBS was used for antibody dilution and washings. Keratinocytes were incubated for 30 min at 4°C first with a monoclonal antibody, either anti-VLA-3 mouse IgG 1:10 or anti-ICAM-1 mouse IgG (Immunotech). After washing, the cells were incubated for 30 min at 4°C with an $F(ab)'_2$ fragment of goat anti-mouse IgG conjugated with FITC 1:15 (Caltag) for detection of VLA-3. For ICAM-1, an amplification method with biotin-streptavidin (Dako) was used. After washing, the cells were fixed in formol 1%. For the detection of TNF-α, a membrane permeabilization technique was used, with anti-TNF-α mouse IgG 1 : 30 (Genzyme) diluted in PBS-saponin 0.5% and an F(ab)'2 fragment of goat anti-mouse IgG conjugated with FITC 1:50 (Caltag). An irrelevant monoclonal antibody of the same isotype as the first antibodies was used as a negative control. Cell fluorescence was detected on a FACScan (Becton-Dickinson). Data were acquired from 2,000 cells in each sample, after elimination of cell debris. The fluorescence signal was read on a logarithmic scale with four log decades and then transformed into a linear scale. The results were analysed on a fluorescence histogram using LYSYS I software (Becton-Dickinson).

In situ *hybridization*

In situ hybridization was performed after 4 and 8 h of keratinocyte incubation with nickel salts. The cold TNF-α DNA probe (800 nucleotides), provided by INSERM U211 (Nantes, France), was first labelled with digoxigenin using a random-priming method. Hybridization was performed by incubating the smears with 10 ng of labelled probe overnight at 42°C in dampness. Revelation was obtained using an anti-digoxigenin monoclonal antibody coupled to alkaline phosphatase (DIG High Prime and DIG Nucleic Acid Detection kit, Boehringer Mannheim). Two types of negative controls were performed: negativation of the labelling by mRNase and omission of the TNF-α probe. The slides were read blindly (two independent investigators) under a Leitz microscope in four different fields (magnification × 40). For expression of the results, a mean was determined for the four fields. Labelling intensity was rated semi-quantitatively: – (negative), + (low intensity), + + (moderate intensity), + + + and + + + + (high intensity).

RESULTS

Immunocytochemistry (Table I)

Post-transcriptional expression of TNFα. Basal expression of TNF-α was initially low (+) for 100% of cells, but increased in the presence of the three salts, reaching maxima at 1.10^{-4} M (+ + + +) for nickel gluconate, 1.10^{-4} M (+ +) for nickel sulphate and 1.10^{-4} M (+ +) and 5.10^{-4} M (+ +) for nickel chloride. With a concentration of 1.10^{-3} M, TNF-α expression was low for all three salts.

ICAM-1 expression. Basal expression of ICAM-1 was non-existent (–), whereas the three nickel salts induced ICAM-1 expression in 100% of cells at different intensities. Maximum expression was observed for nickel gluconate at 5.10^{-5} M (+ +), 1.10^{-4} M (+ +) and 5.10^{-4} M (+ +), for nickel sulphate at 1.10^{-4} M (+ +) and 5.10^{-4} M (+ +), and for nickel chloride at 5.10^{-5} M (+ +) and 1.10^{-4} M (+ +). With a concentration of 1.10^{-3} M, expression was no longer detectable for any of the salts.

VLA-3 expression. Basal expression of VLA-3 was moderate (+ +) in 100% of cells. All three nickel salts enhanced the intensity of VLA-3 expression, with maxima for nickel gluconate at 5.10^{-5} M (+ + +), 1.10^{-4} M (+ + +) and 5.10^{-4} M (+ + +), for nickel sulphate at 5.10^{-5} M (+ + +) and 1.10^{-4} M (+ + +), and for nickel chloride at 1.10^{-4} M (+ + +). With the concentration of 1.10^{-3} M, expression was low (+) for nickel gluconate and sulphate and non-existent for nickel chloride.

Keratinocyte activation was maximal with 1.10^{-4} M, regardless of the salt studied. This concentration was then used in flow cytometry to differentiate the three salts quantitatively.

Transcriptional expression of TNF-α

Compared to the control medium which had low labelling intensity (+), the mRNA expression of TNF-α was enhanced at 8 h with all three nickel salts, with no significant differences in intensity (70 to 95% of cells labelled with an intensity of + + +).

Modulation by zinc gluconate of VLA-3 expression induced by nickel gluconate 1.10^{-4} M (Table II)

Zinc gluconate alone, at a concentration of 0.9 or 9 μg/ml, did not modify VLA-3 expression as compared to salt-free control medium. However, the 9 μg/ml concentration caused a 56.6% reduction ($p < 0.01$) in the VLA-3 expression induced by nickel gluconate.

DISCUSSION

Our study was the first to compare the inflammatory activity of nickel gluconate, sulphate and chloride *in vitro*. A toxic effect is shown for all three salts at 1.10^{-3} M. This toxic effect, in the

Table I. *Flow cytometry study of modulations in keratinocyte expression of ICAM-1, TNF-α and VLA-3 by the three nickel salts at 1.10^{-4} M*

	ICAM-1 MFI	TNF-α MFI	VLA-3 MFI
Control	2.63 ± 1.12	8.64 ± 0.92	132.26 ± 26.70
Nickel gluconate 1.10^{-4} M	15.72 ± 1.39 (**)	30.94 ± 2.33 (*)	220.58 ± 9.70 (*)
Nickel sulphate 1.10^{-4} M	12.58 ± 1.73 (**)	26.35 ± 0.60 (**)	180.86 ± 19.37
Nickel chloride 1.10^{-4} M	13.96 ± 0.17 (**)	26.49 ± 4.48 (*)	148.52 ± 8.53

MFI: Mean fluorescence intensity expressed on a logarithmic scale. NB: Student's *t*-test, $*p < 0.05$, $**p < 0.01$.

Table II. *Flow cytometry study of modulations by zinc gluconate of keratinocyte expression of VLA-3 induced by nickel gluconate 1.10^{-4} M*

	VLA-3 MFI
Control	99.83 ± 13.95
Zinc gluconate 0.9 μg/ml	81.55 ± 11.99
Zinc gluconate 9 μg/ml	74.03 ± 10.10
Nickel gluconate 1.10^{-4} M	278.70 ± 26.55
Zinc gluconate 0.9 μg/ml and then nickel gluconate 1.10^{-4} M	264.36 ± 25.58
Zinc gluconate 9 μg/ml and then nickel gluconate 1.10^{-4} M	120.90 ± 1.02 (**)

MFI: Mean fluorescence intensity expressed on a logarithmic scale. NB: Student's *t*-test, *$p < 0.05$.

absence of cell death (>95% cell viability in tryptan-blue staining), can be attributed in part to the greater membrane permeability of keratinocytes when the nickel ion concentration is too high (3). For other concentrations, the expression of ICAM-1, VLA-3 and TNF-α was increased with the three nickel salts. Although the flow cytometry study showed no significant differences between the three salts, nickel gluconate was generally prevalent over the other two, and these results are confirmed with immunochemistry. This prevalence suggests the involvement of chemical factors, since nickel gluconate is the only salt in which the nickel ion is found in complex form, which may favour its penetration within keratinocytes. The absence of a significant difference between the three nickel salts suggests that it is essentially the nickel ion (and not the accompanying salt) which is responsible for keratinocyte activation. The mechanism of nickel action is poorly understood. The nickel ion supposedly stimulates the lipoxygenase pathway, resulting in the generation of hydrogen peroxides (6) and activation of protein kinase C (7).

Concerning inflammation markers, our study showed that nickel salts significantly enhance TNF-α production and ICAM-1 and VLA-3 expression.

Immunocytochemistry and flow cytometry showed a constitutive intrakeratinocyte production of TNF-α, which plays a pleiotropic role in inflammatory processes (8, 9). For the three nickel salts, the increase in this cytokine noted at the protein level was confirmed at the transcriptional level, with a maximum stimulation at 8 h (data not shown). However, contrary to the protein level, where nickel gluconate induced the strongest label, no difference was noted between the three salts at the transcriptional level. This fact may be related to the lack of sensitivity of the hybridization technique to detect low differences of modulation between salts.

In agreement with other studies (2), our work showed that keratinocytes did not express ICAM-1 constitutively, but that nickel salts significantly induced its expression. ICAM-1 plays a fundamental role in inflammatory reactions, in particular through lymphocyte-keratinocyte interactions (10, 11).

Finally, we have shown that keratinocytes expressed VLA-3 constitutively and that stimulation by nickel salts, and more specifically nickel gluconate at 1.10^{-4} M, increased the expression of this integrin. VLA-3 is expressed constitutively by keratinocytes in basal layers of the epidermis (12) and its ligands are constituents of the extracellular matrix (13). In some inflammatory dermatoses, such as contact eczema, lichen and psoriasis, the expression of this integrin is increased mainly in the suprabasal layers of the epidermis (5). A new role for VLA-3 has recently been suggested in intercellular adhesion events. In fact, the absence of polarization of the α_3 chains toward the extracellular matrix proteins of the basement membrane (14) suggests that VLA-3 is mainly involved in intercellular reactions of basal cells of the epidermis and to a lesser extent in adhesion and anchoring to the basement membrane. Thus, an overexpression of this integrin in local inflammatory events could play a role in the persistence of certain cells, such as T-lymphocytes, at the epidermal level. The results obtained with VLA-3 suggested that this integrin might be of interest as an inflammation marker. Our experiments showed that zinc gluconate produced an inhibitory effect on keratinocyte expression of VLA-3. Previous studies had shown that zinc can reduce the expression induced by nickel in some adhesion molecules (ICAM1) (15). The zinc acted to prevent the formation of free radicals through a competition mechanism with nickel. It would appear to stabilize membrane lipoprotein components by forming mercaptides with the thiol groups of membrane proteins (16), reduce lipid peroxidation by increasing superoxide dismutase activity (17), and, finally, take up the free radicals that form by enhancing the synthesis of metalloproteins (18).

In conclusion, our study not only confirmed the inflammatory properties of nickel but also allowed us to define the concentration of a nickel salt (nickel gluconate, 1.10^{-4} M) which in our culture conditions produced maximal keratinocyte activation. It also suggested the potential interest of VLA-3 as an inflammation marker, in addition to ICAM-1, since the expression of both of these molecules is reduced by a zinc salt.

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